



PATENT
2786-0177P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: Liat MINTZ et al. Conf.: 2695
Appl. No.: 09/884,050 Group: 1646
Filed: June 20, 2001 Examiner: JIANG, D.
For: NOVEL VEGF NUCLEIC ACID AND AMINO ACID
SEQUENCES

L E T T E R

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

December 31, 2003

Sir:

Attached, please find an executed declaration under 37 C.F.R. § 1.132 by Zoya Gluzman-Poltorak and Belly Koren. This executed declaration is to replace the unexecuted declaration submitted with the response filed October 31, 2003.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

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Attachment(s)



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors	Liat MINTZ, Kinneret SAVITZKY and Sharon ENGEL
Serial No.	09/884,050
Filed	20/JUN/2001
Title	Variable vegf/novel nucleic acid and amino acid sequences
Group Art Unit:	1646
Examiner:	JIANG, DONG

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION

(1) SIR:

We, Zoya Gluzman-Poltorak and Belly Koren, do hereby declare:

1. I, Zoya Gluzman-Poltorak, hold a Ph. D. degree from the Dept. of Biology, Technion – Israel Institute of Technology, Haifa, Israel. The subject of my thesis is characterization of Neuropilin-2 – a novel VEGF receptor. The subject of my M. Sc. thesis is characterization of VEGF145- a novel VEGF splice variant.

Since the year 2000, I have been working at the Molecular Cardiology Laboratory, Carmel Medical Center, Haifa, Israel, as a Project Manager.

I am experienced in the following fields: state-of-the art molecular biology techniques, construction of recombinant mammalian and insect expression systems and tissue culture techniques; DNA and RNA techniques; protein expression, purification and analysis; cell biology and signal transduction research; polyclonal antibodies production and purification; histology techniques including immunostaining and in-situ hybridization; adeno- and retroviral vector construction, production and purification for human gene therapy applications; animal models establishment (pig, sheep, mouse); preclinical trials conductance; and regulatory processes. A copy of my Curriculum Vitae is attached.

2. I, Belly Koren, have an M. Sc. degree from The Faculty of Food Engineering and Biotechnology, Technion - Israel Institute of Technology. I work at the Molecular Cardiology laboratory, Carmel Medical Center, Haifa, Israel. A copy of my Curriculum Vitae is attached.

3. The aim of the experiments described below was to investigate the properties of VEGF₁₁₄ - a novel VEGF isoform. In order to characterize VEGF₁₁₄ it was over-expressed as a protein in endothelial cells (EC) and smooth muscle cells (SMC) using adeno- and retroviral expression systems and its biological properties were compared with those of the known VEGF₁₆₅ isoform.

METHODS

I. Generation of recombinant viral vectors:

Generation of recombinant Adenoviral vectors encoding *VEGF*₁₁₄ gene

The recombinant adenoviral vector expressing the human VEGF₁₁₄ gene was constructed by a modified AdEasy protocol (Vogelstein B. PNAS 1998). A 426bp BamHI-XhoI fragment of VEGF₁₁₄ cDNA was inserted into the BglII- XhoI sites in the pAdShuttle-CMV vector under the control of the CMV promoter. The shuttle vectors were linearized by PmeI digestion and purified by Qiaquick gel extraction kit. The linearized shuttle vector and pAdEasy-1 were co-transformed into competent BJ5183 cells by electroporation. Positive clones containing the recombinant adenoviral vectors were selected according to PCR and restriction map analysis. The recombinant adenoviral plasmids were linearized by PacI digestion, purified and transfected into 293 cells using Lipofectamine 2000 (Gibco BRL, USA). Seven days after transfection CPE (cytopathic effect) occurred and 100% of the cells expressed Green Fluorescent Protein (GFP). The cells were harvested and viral extracts were further amplified in 293 cells. The viral stock titer was determined by serial dilution assay in 293 cells and was $\sim 10^{11}$ pfu/ml. The expression of the transgene was confirmed by Western analysis of conditioned media from the infected cells.

Construction of retroviral vectors for expression of *VEGF*₁₁₄ or co-expression of *VEGF*₁₁₄ and GFP

The recombinant retroviral vector LXSN-VEGF encoding the human VEGF gene was constructed by inserting the human VEGF₁₁₄ cDNA 426bp EcoRI-XhoI fragment into the EcoRI-XhoI site of plasmid pLXSN (# K1060-B Clontech, USA) under the control of Mo-MULV 5' long terminal repeat (LTR).

The recombinant retroviral vectors expressing the human VEGF₁₁₄ and /or the EGFP genes were constructed by cloning into pLXSN plasmid in two steps. First, an IRES-EGFP EcoRI-HpaI fragment (1400 bp) excised from pIRES2-EGFP (#6029-1 Clontech) was inserted into EcoRI-HpaI sites in pLXSN for construction of the control plasmid, pLXSN-IRES-EGFP. The second step was construction of pLXSN-VEGF₁₁₄-IRES-EGFP by cloning of a human VEGF₁₁₄ EcoRI-Xho I fragment (426 bp) into the EcoRI-Sal I sites in pLXSN-IRES-EGFP. The expression cassettes are regulated by the Mo-MULV 5' long terminal repeat (LTR).

Generation of pseudotyped recombinant retroviral vectors encoding *VEGF*₁₁₄

For retroviral vector production, pLXSN-VEGF₁₁₄-IRES-GFP vector or pLXSN-VEGF₁₁₄ was transfected into 293FLYA packaging cells using Lipofectamine (Gibco BRL, USA). After 48 hours, supernatant from confluent cultures of viral producer cells was collected, filtered (0.45 μ m) and added to 293 FLYGALV packaging cells. Transduced cells were grown under G418 selection (400 μ g/ml) and individual colonies were collected and screened for EGFP

expression, using an inverted fluorescent microscope, and VEGF₁₁₄ expression by Western analysis of transduced cell-conditioned medium. The viral titer of each colony was determined via transduction of TE671 cells and the titers of $\sim 10^6$ pfu/ml were obtained. The colonies with the highest-titers were selected and supernatant was collected freshly for transduction of EC and SMC.

II. Verifying transgene expression after gene transfer

Cell culture

Endothelial cells (EC) were isolated from human saphenous veins (HSVEC), and cultured on gelatin-coated dishes in M20 containing M-199 Medium (Biological Industries, Israel) supplemented with 20% FCS, 2mM L-Glutamin, 100 units/ml penicillin, and 0.1 mg/ml streptomycin, 100µg/ml Heparin (Sigma) and 2ng/ml bFGF (Enco). Human EC were identified by immunohistochemistry analysis with anti Von-Willebrand factor specific antibodies (Zymed, USA). Smooth muscle cells (SMC) were cultured by explant outgrowth from human saphenous veins (HSVSMC) and left internal mammary arteries (HLSMC). Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Biological Industries, Israel) supplemented with 10% human serum, 2 mM L-Glutamin, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 2ng/ml bFGF. SMC were identified by immunohistochemistry analysis using specific anti- α smooth muscle actin antibodies (Zymed, USA).

The packaging cell lines 293-FLYA, 293-FLYGALV and TEFLYGA (obtained from Dr F.L. Cosset - Lyon, France) were grown in DMEM supplemented with 10% FCS, 2mM L-Glutamine, 100 units/ml penicillin, 0.1mg/ml streptomycin, 6µg/ml blasticidin and 6µg/ml phleomicin.

Infection of EC and SMC with recombinant adenovirus vectors

Endothelial cells and smooth muscle cells were infected with recombinant adenoviral vectors as follows: the cells were seeded at 70% confluence on fibronectin pre-coated plates (4.5µg/ml) 20 hours prior to infection and grown in complete medium (M20). At the day of infection the medium was replaced with fresh M199 (without serum) and the recombinant virus was added at Multiplicity of Infection of 1000 (MOI=1000). The cells were incubated for 90 minutes with gentle tilt every 20 minutes. After infection the virus-containing medium was replaced with complete medium (M20).

Transduction of EC and SMC with recombinant retroviral vectors

Transduction of endothelial cells and smooth muscle cells with the retroviral vectors was performed as followed: EC (passage 4-9) were seeded (10^5 cells per 35-mm well) in fibronectin-coated plates (4.5µg/ml) and grown in complete medium for 24 hours. One hour prior to transduction, the medium was replaced with serum free M199 containing 0.1mg/ml of the cationic polymer DEAE-dextran (Sigma). After pre-conditioning, the cells were washed three times with phosphate-buffered saline (PBS). Transduction was performed by incubation of the cells for 4 hours with supernatants containing viruses freshly collected and filtered (0.45µ) from the virus-producing packaging cell lines. At the end of the incubation the medium was replaced with fresh M20 medium.

III. VEGF₁₁₄ over-expression by infected EC and SMC

Western blot analysis

VEGF₁₁₄ protein expression by adenoviral- or retroviral-infected EC and SMC was detected by ELISA or Western blot analysis of the conditioned medium. 24 hours post infection the medium was changed to serum free medium and cells were grown for an additional 48 hours.

For Western blot analysis the samples of the conditioned medium (30µl) were separated on 8% SDS polyacrylamide gel under reducing conditions, and electrotransferred to nitrocellulose membrane (Shleicher & Schuell). The blots were blocked with 0.1% skim milk in TBS containing 0.3% tween-20 (TBST) for 1 hour at room temperature using gentle agitation. The blots were incubated with primary antibody diluted in blocking solution for 2 hours at room temperature. 1:500 dilution of polyclonal rabbit anti-VEGF antibody (#SC 152 Santa- Cruz, USA) was used for VEGF₁₁₄ detection. Following the incubation the blots were washed three times with TBST and incubated for 1 hour at room temperature with anti rabbit peroxidase-conjugate antibody (Sigma) diluted 1:7000 in TBST. After three washes with TBST, bound antibody was visualized using ECL reagents (Sigma) and exposed to X-ray film.

To detect VEGF₁₁₄ protein concentration in the conditioned medium of viral infected cells, a Human VEGF ELISA kit (Oncogene, Cat# QIA51) was used according to the manufacturer's instructions.

IV. Physiological Effects of VEGF₁₁₄ gene transfer

Proliferation assay with conditioned medium of rAd VEGF₁₁₄ infected EC

Conditioned medium containing VEGF₁₁₄ was collected as follows: EC (passages 5-11) were seeded in 6-well plates at 150,000 cells/well and infected with rAd VEGF₁₁₄, rAd VEGF₁₆₅-GFP or rAdGFP. Non-infected cells were used as an additional control group. 24 hrs after infection the medium was changed to serum free medium and the cells were grown for an additional 48 hours. The conditioned medium was collected and used for the following proliferation assay.

To perform the proliferation assay, EC were seeded at 2×10^4 cells/ well in 24 well plates pre-coated with fibronectin (4.5µg/ml) in M199 containing 5% FCS. The assay was performed in triplicates. Increasing volumes of conditioned medium containing VEGF₁₁₄, VEGF or control were added each other day. Proliferation rate was detected 6-7 days after infection by cell counting.

Proliferation assay with recombinant retro VEGF₁₁₄ transduced EC

EC (passages 5-11) were transduced with recombinant retro VEGF₁₁₄-GFP, VEGF₁₆₅-GFP or GFP viruses. Non-infected cells served as an additional control group. Retrovirus transduced cells were isolated by selection with 250 µg/ml of G418. After selection the cells were seeded at the concentration 6×10^5 cells/well in 6-well plates pre-coated with fibronectin (4.5µg/ml) in M199 containing 20% FCS. The assay was performed in duplicate. Proliferation rate was detected 7 and 10 days after seeding by cell counting.

In-vitro angiogenesis assay – sprouting assay in collagen

In-vitro angiogenesis was examined using endothelial cell (EC), smooth muscle cell (SMC), and mixed EC and SMC coculture sprouting from spheroids in collagen three-dimensional matrix. The generation of spheroids was performed as described by Korff T. and Augustin H. (JBC 1998). EC were tagged with DiI-291 red fluorescent marker prior to mixing. 750 cells (EC or SMC) per single culture spheroid or 375 cells from each type (EC and SMC) for coculture spheroid were suspended in culture medium containing 0.25% (w/v) carboxymethylcellulose, seeded in nonadherent round-bottom 96-well plates (Nunc, Denmark). During 24 h incubation at 37°C, 5% CO₂ the suspended cells form a single spheroid per well of defined size and cell number. The spheroids generated were then embedded in collagen gels. A collagen stock solution was prepared prior to use by mixing 8 vol acidic collagen extract of rat tails (equilibrated to 2 mg/ml, 4°C) with 1 vol 10 x M199 (Gibco BRL, USA); 1 vol neutralization solution containing 0.34 N NaOH and 7.5% NaHCO₃ to adjust the pH to 7.4. This stock solution (0.5 ml) was mixed with 0.5 ml room temperature medium M199 with 40% human serum containing 0.5% (w/v) carboxymethylcellulose to prevent sedimentation of spheroids before polymerization of the collagen gel. The spheroids (20-30) containing gel was rapidly transferred into prewarmed 24-well plates and allowed to polymerize. The gels were incubated at 37°C, 5% CO₂ and documented using a digital video camera (DXM1200 Nikon, Japan).

Apoptosis assay

The percentage of cells within a population that are actively undergoing apoptosis may be determined by assessing the binding of Annexin V-PE to phospholipid phosphatidylserine (PS). The assay relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing PS to the external environment. Annexin V is a Ca²⁺-dependent phospholipid-binding protein that has a high affinity for PS, and is useful for identifying apoptotic cells with exposed PS, and binds to cells with exposed PS. Annexin V maybe conjugated to fluorochromes such as Phycoerythrin (PE). This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometry analysis of cells that are undergoing apoptosis. 7-Amino-actinomycin (7-AAD) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD. Cells that stain positive for Annexin V-PE and negative for 7-AAD are undergoing apoptosis. Cells that stain positive for both Annexin V-PE and 7-AAD are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both Annexin V-PE and 7-AAD are alive and not undergoing measurable apoptosis.

Retroviral vector transduced HSVEC were seeded onto gelatinized 6-well plates (2*10⁵ cells/well) in M199 medium supplemented with 20% (v/v) fetal calf serum (FCS) and incubated for 24 h.

In order to induce apoptosis, the cells were "pulse" exposed to H₂O₂: the medium was changed to 1ml of modified-PBS + 1507M H₂O₂ for 1 hour at 37° and then changed to M199 medium supplied with 2% FCS and incubated for 24 h.

All floating cells and all adherent cells were collected to the same tube after trypsinization (0.05% trypsin-EDTA). The cells were then pelleted, washed with ice-cold PBS, and resuspended in 1x binding buffer at a concentration of 1*10⁶ cells/ml.

Annexin V-PE and 7-AAD (ready-made solutions) were added to the cells, mixed and incubated for 15 minutes at RT in the dark. Finally, 1x binding buffer was added to a final volume of 0.5ml. Analysis by a FACScan flow cytometer equipped with a doublet discriminating module was performed within 1 hour.

For each checked cell we used two controls. One of unstained cells and the other of Annexin V stained alone, in order to reduce the GFP signal that interferes with the PE signal.

RESULTS

Sequence analysis of VEGF₁₁₄ cDNA

Both strands of the VEGF₁₁₄ cDNA were sequenced and the nucleotide sequence was compared with published VEGF sequence. VEGF₁₁₄ cDNA consists of exons 1-5 which contain information required for the recognition of the known VEGF receptors VEGFR1 and VEGFR2 and are present in all VEGF isoforms (Keyt, B.A., 1997). The VEGF₁₁₄ cDNA lacks exons 6 and 7 encoding heparin binding domains and present in VEGF₁₄₅ and VEGF₁₆₅ respectively. VEGF₁₈₉ contains both of the heparin binding domains. The VEGF₁₁₄ cDNA also lacks exon 8 which exists in all other known VEGF isoforms. To conclude, VEGF₁₁₄ cDNA resembles the structure of VEGF₁₂₁ cDNA without exon 8. We have not found any mutation in the VEGF₁₁₄ cDNA.

VEGF₁₁₄ expression in EC and SMC

To study the properties of VEGF₁₁₄, VEGF₁₁₄ cDNA was expressed in EC and SMC using adenoviral and retroviral expression systems. Transgene expression was verified by detection of fluorescence from GFP. Transduction of EC and SMC by retroviral vector encoding VEGF₁₁₄-GFP demonstrated the production of the GFP protein (figure 1).

VEGF₁₁₄ protein expression was examined by Western blot analysis (~18 kD protein corresponding to the VEGF₁₁₄ protein) after infection of EC with an adenoviral vector (figure 2) or retroviral vector (figure 3) encoding VEGF₁₁₄. To detect VEGF₁₁₄ protein concentration in the conditioned medium of infected cells we used a Human VEGF ELISA kit (Oncogene, Cat# QIA51). The concentrations of VEGF₁₁₄ and VEGF₁₆₅ proteins were 2.7ng/μl and 0.3ng/μl respectively.

VEGF₁₁₄ biological activity

The biological activity of the recombinant VEGF₁₁₄ was tested using a proliferation assay, an in-vitro angiogenesis assay and an apoptosis assay.

The proliferation assays were performed with 1) naive EC by addition of conditioned medium from rAd VEGF₁₁₄ infected EC or 2) with recombinant retroVEGF₁₁₄ transduced EC. In both kinds of experiments VEGF₁₁₄ induces proliferation of EC, but it was less potent than VEGF₁₆₅ (figures 4 and 5).

The effects of VEGF₁₁₄ expression was tested on EC sprouting from spheroids in collagen three-dimensional matrix. Endothelial cells over expressing VEGF₁₁₄ after retroviral gene transfer had no effect on EC sprouting while EC over expressing VEGF₁₆₅ exhibited an increased sprouting (figure 6A). The same results were obtained from co-culture experiments with retrovirus GFP transduced EC and VEGF₁₆₅/GFP or VEGF₁₁₄/GFP retro transduced SMC (figure 6B).

To examine whether over-expression of VEGF₁₁₄ or VEGF₁₆₅ in EC protects the cells from undergoing apoptosis Annexin V-PE staining, which can identify

apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation, was used. FACScan flow cytometer analysis was performed to detect Annexin V-PE staining. As shown in figure 7, both VEGF₁₁₄ and VEGF₁₆₅ protected EC from apoptosis, though VEGF₁₆₅ was more potent than VEGF₁₁₄.

CONCLUSIONS

From the described study it can be concluded that expression of VEGF₁₁₄ has definite effects on EC proliferation and apoptosis, but it is less potent than VEGF₁₆₅. In addition to the proliferation and apoptosis experiment, a 3-dimensional *in-vitro* angiogenesis assay was performed; VEGF₁₁₄ had no effect on EC sprouting. This VEGF₁₁₄ exhibits two of the three assayed bioactivities of VEGF₁₆₅, although with less potency.

4. The above-described experiments were conducted at the request of Dr. Kinneret Savitsky, who is listed as a co-inventor of the subject matter of the above-identified patent application. The above experiments were performed by Dr. Zoya Gluzman-Poltorak and Mrs. Belly Koren under the supervision of Dr. Moshe Flugelman, who has been informed of the text of the specification and claims of the above-identified patent application.
5. I declare further that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Zoya Gluzman
Zoya Gluzman-Poltorak

Koren Belly
Belly Koren

Dated this 26th day of Nov., 2003

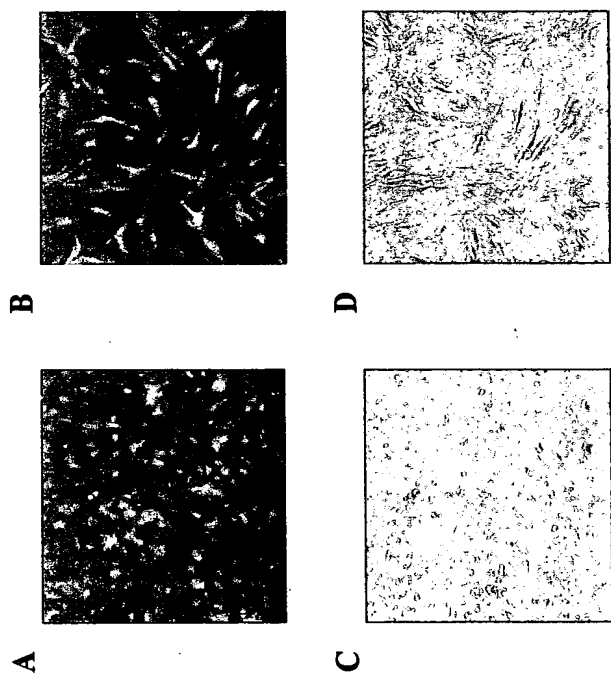


Figure 1: Transduction of EC and SMC with retro VEGF₁₁₄-GFP. HSVEC (A and C) and HVSVMC (B and D) were transduced with retro VEGF₁₁₄-GFP and visualized by either fluorescent inverted microscope (A and B) or by light inverted microscope (C and D).



V₁₁₄ GFP control V₁₆₅
 1 2 3 4

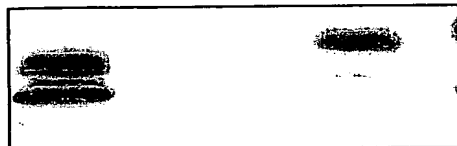


Figure 2: Western blot analysis of VEGF₁₁₄ expression by rAd infected EC.

EC were infected by adenoviral vectors as previously described. 24

hours post infection the medium was changed to serum free medium and cells were grown for additional 48 hours. Samples of the conditioned medium (30μl) were loaded on 12% SDS polyacrylamide gel, transferred to nitrocellulose membrane and the blots were incubated with anti-VEGF antibody. Following exposure to a peroxidase-conjugate secondary antibody the blots were developed with ECL reagents and exposed to X-ray film.

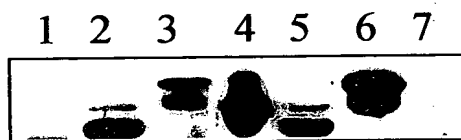
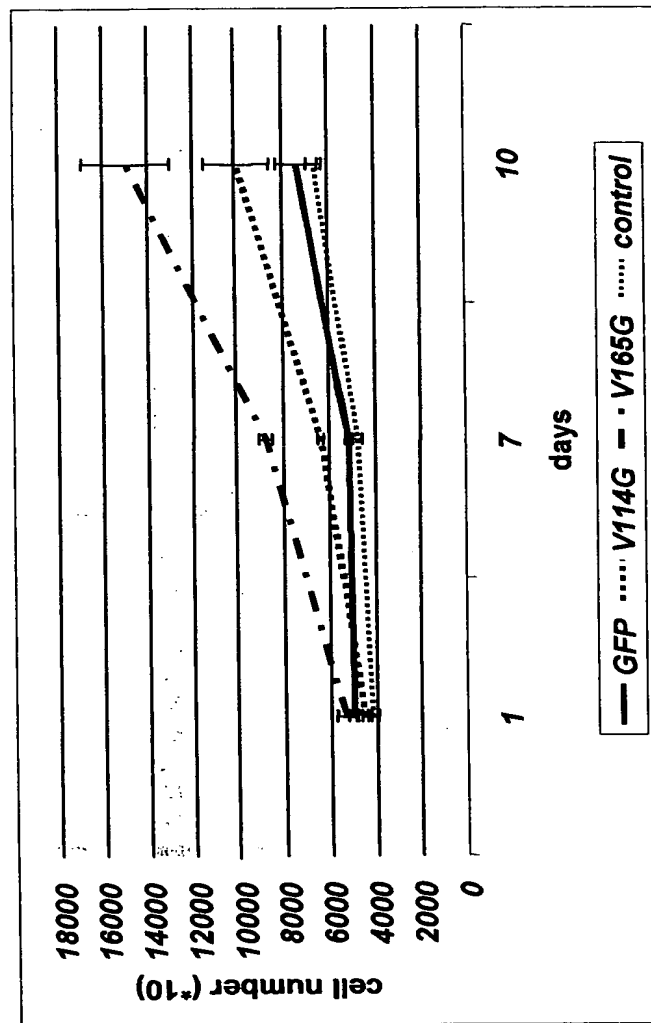
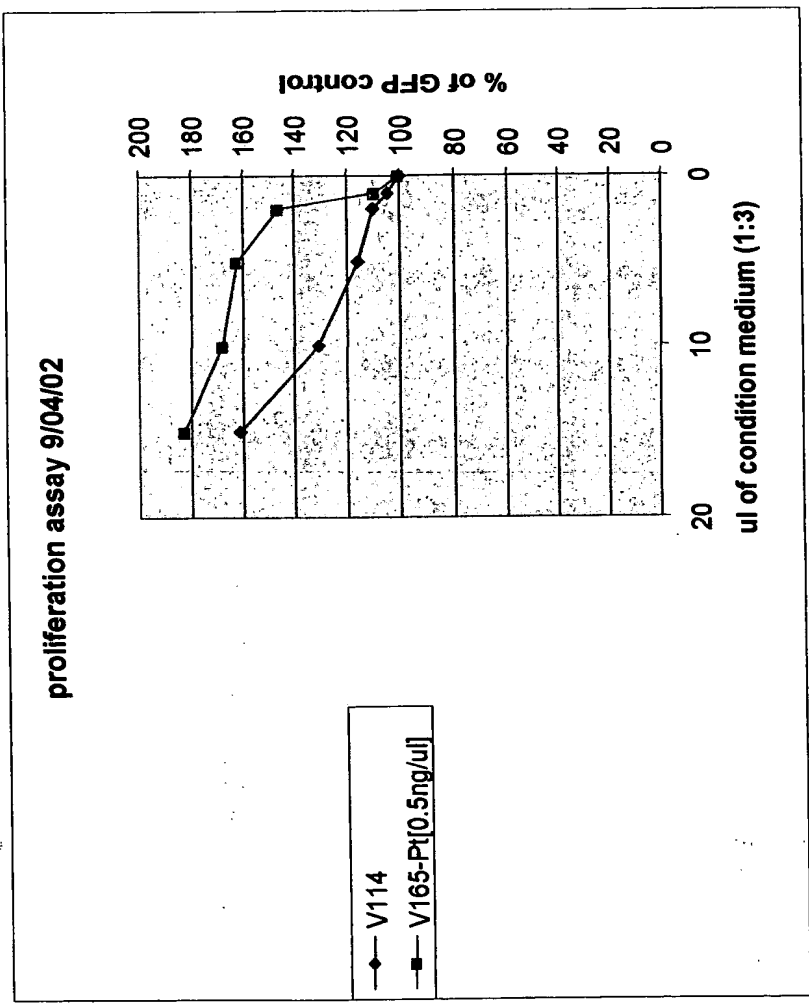


Figure 3: Western blot analysis of VEGF₁₁₄ expression by retro transduced EC and SMC.

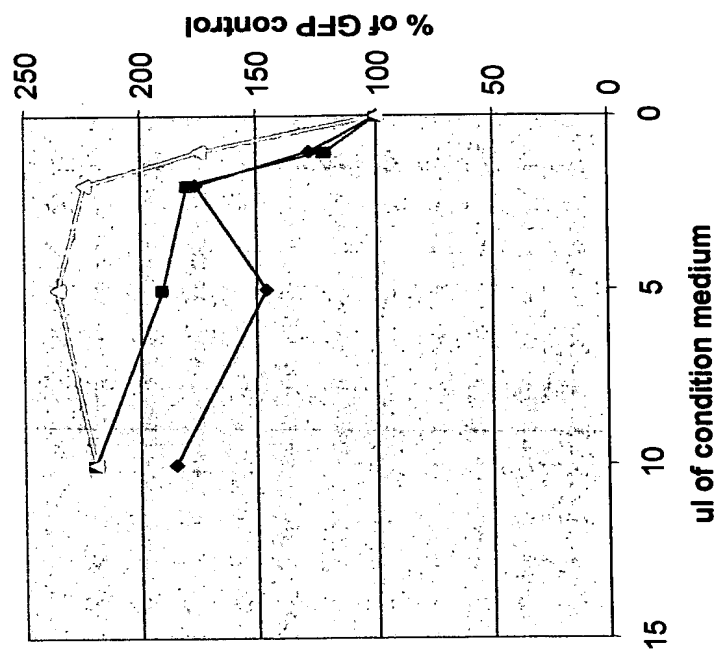
EC and SMC were transduced by retro vectors as previously described. 24 hours post infection the medium was changed to serum free medium and cells were grown for additional 48 hours. Samples of the conditioned medium (30μl) were loaded on 12% SDS polyacrylamide gel, transferred to nitrocellulose membrane and the blots were incubated with anti-VEGF antibody. Following exposure to a peroxidase-conjugate secondary antibody the blots were developed with ECL reagents and exposed to X-ray film. Lanes are as follows: 1. Control non-transduced SMC; 2. retro VEGF₁₁₄ transduced SMC; 3. retro VEGF₁₆₅ transduced SMC; 4. positive control of VEGF₁₆₅; 5. retro VEGF₁₁₄ transduced EC; 6. retro VEGF₁₆₅ transduced EC; 7. Control non-transduced EC

figure 4: Proliferation assay with recombinant retro VEGF₁₁₄ transduced EC



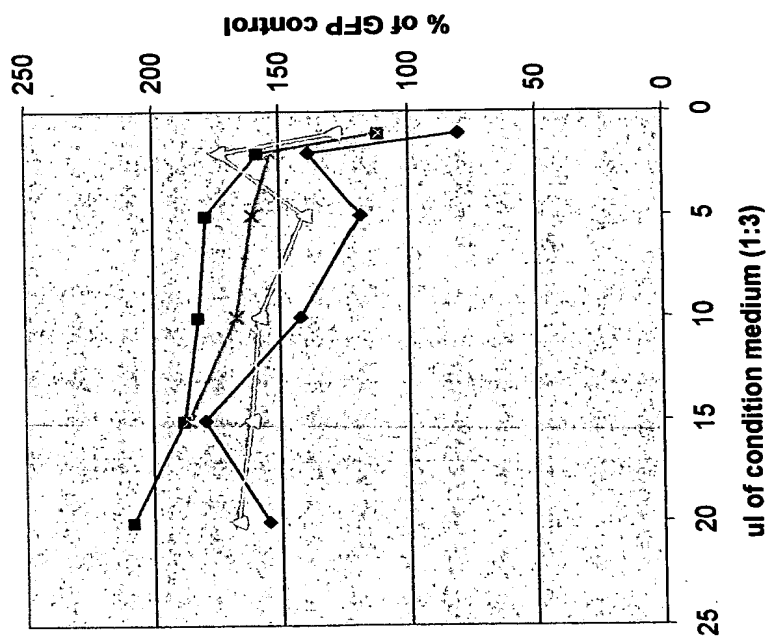


Proliferation assay 25/03/02



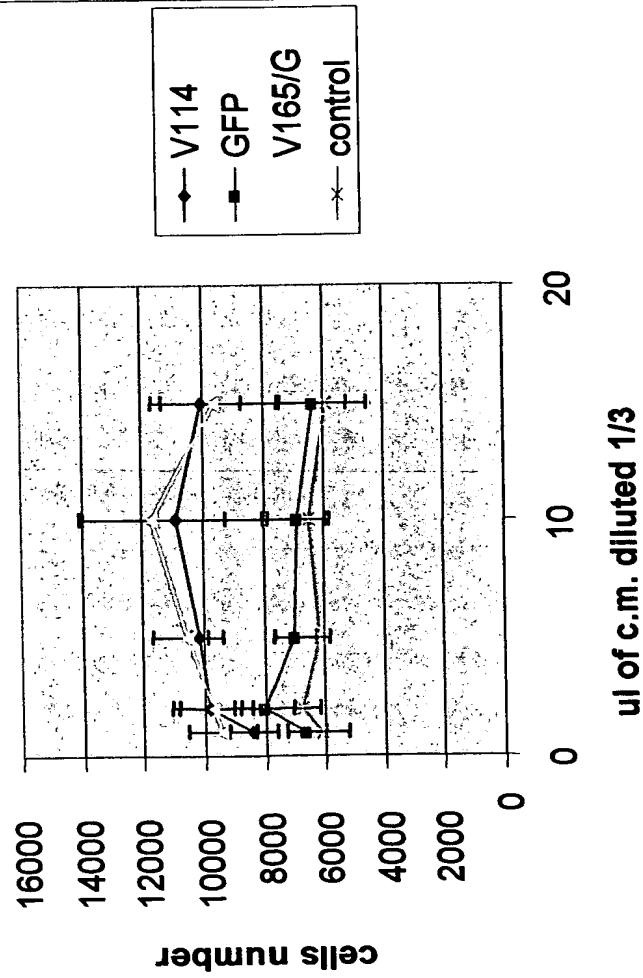
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—■— V165/G
—▲— V165-Pt[2ng/ul]

proliferation assay 2/05/02

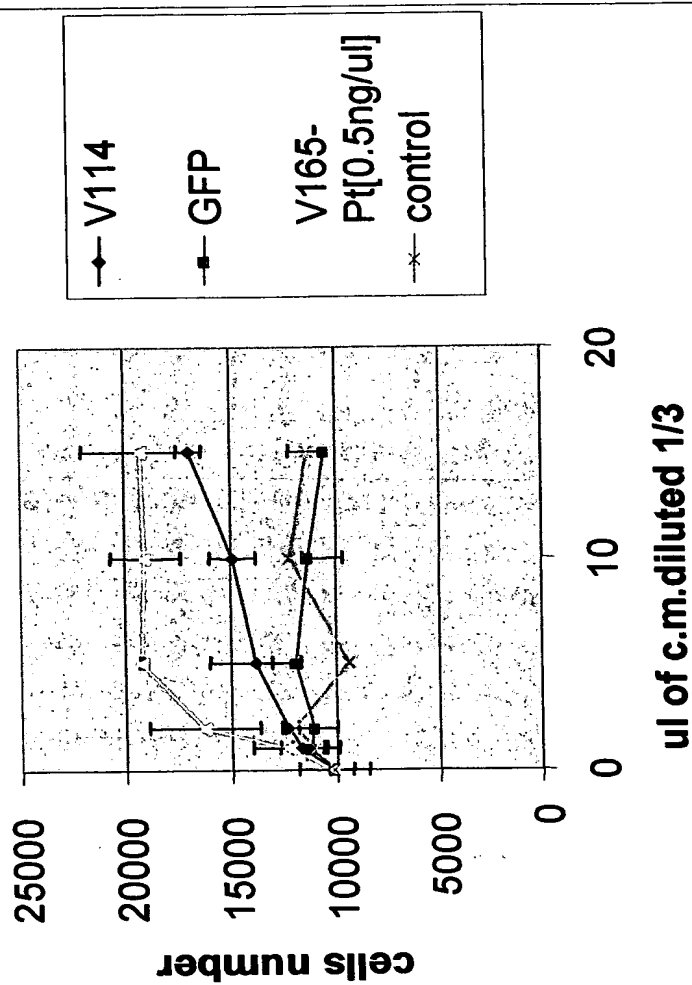


—◆— V114
 —■— V165/G
 —▲— V165-Pt[0.5ng/ul]
 —×— HGF/GFP

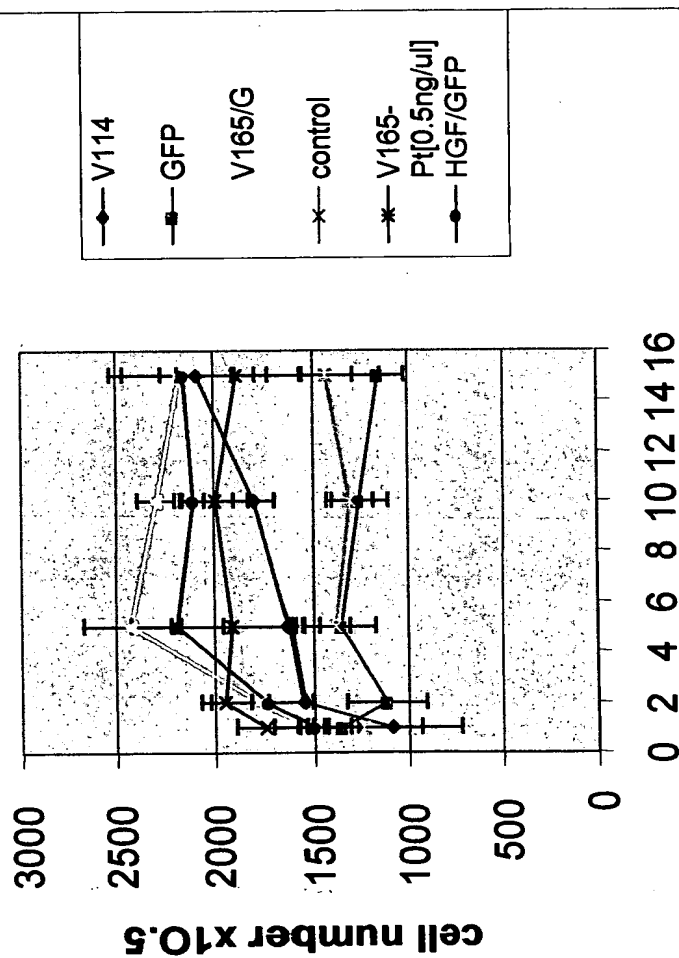
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PROLIFERATION ASSAY 2/05/02



proliferation assay 25/03/02

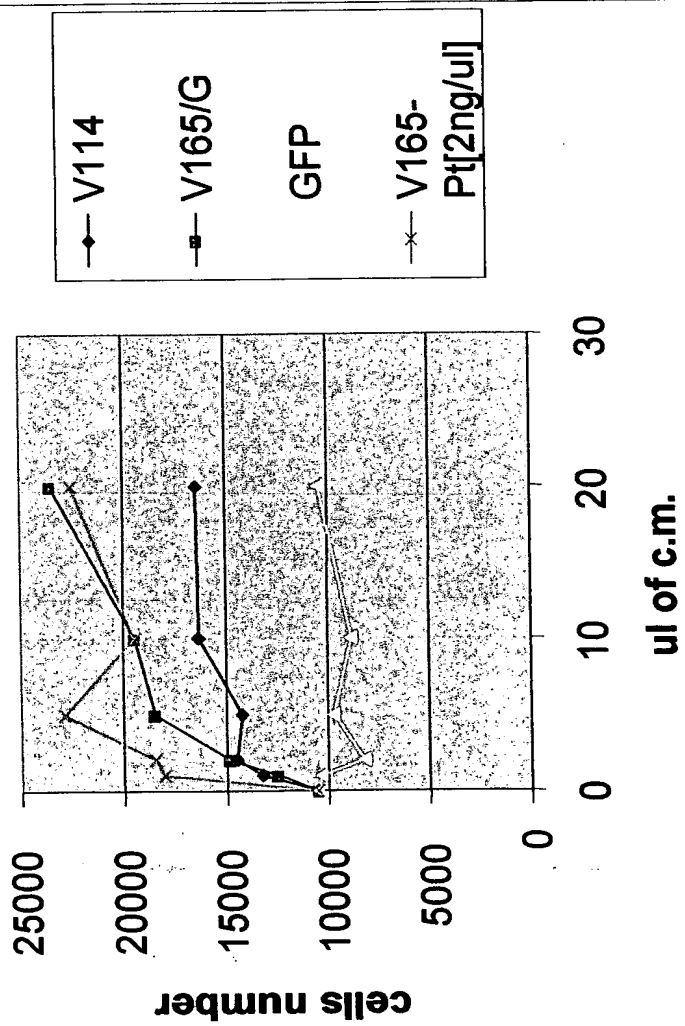
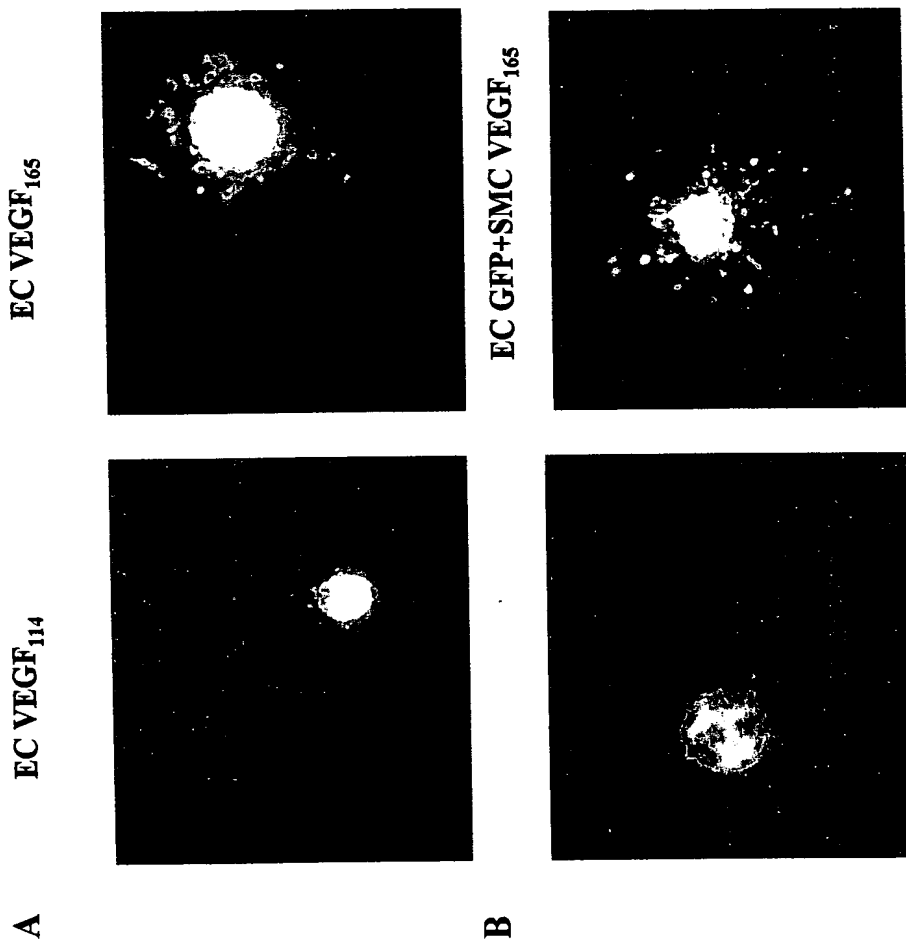


figure 6: In-vitro angiogenesis assay.- sprouting assay in collagen



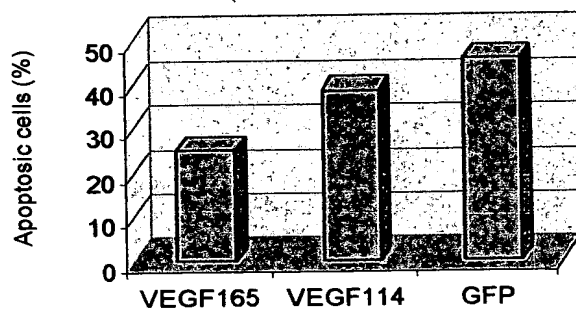


Figure 7: Apoptosis rate in recombinant retro transduced EC

EC were transduced with recombinant retro VEGF₁₁₄-GFP, VEGF₁₆₅-GFP or GFP viruses. The assay was performed as described in methods. FACSscan flow cytometer analysis was performed to detect Annexin V-PE staining.